Monoxenic Mass Production of the Entomogenous Nematode Neoaplectana carpocapsae Weiser on Dog Food/Agar Medium

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ABSTRACT

An economical mass production method for rearing the entomogenous nematode, *Neoaplectana carpocapsae* Weiser, using dog food/agar medium, is described. Infective juveniles are produced monoxenically (with one associated micro-organism) and are separated from the noninfective stages. This medium produced over 125 million infective juveniles per week from 100 petri dishes at a total cost of 28 cents per million nematodes. About 100,000 juveniles were produced per gram of dog food/agar medium. Plastic tissue culture flasks (150 cm^2) and petri dishes (150 by 15 mm) provided proper conditions for storage for 180 days.

KEYWORDS: Biological control, *Neoaplectana carpocapsae*, entomogenous nematodes, nematode propagation and storage, nematode rearing media.

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MONOXENIC MASS PRODUCTION OF THE ENTOMOGENOUS NEMATODE, *NEOAPLECTANA CARPOCAPSAE* WEISER, ON DOG FOOD/AGAR MEDIUM[1]

By Arnold H. Hara, James E. Lindegren, and Harry K. Kaya[2]

INTRODUCTION

The entomogenous nematode, *Neoaplectana carpocapsae* Weiser, shows considerable promise as a biological control agent because it infects a wide range of insect pests and can be mass produced on artificial media. Infective third-stage juveniles (=dauer juveniles) have been used against a variety of pest insects under field conditions with reduction in pest populations (*13*).[3]

Economical mass production and storage methods are needed for large-scale field testing of *N. carpocapsae*. Various artificial media have been used to rear this nematode. Some examples are pork kidney/peptone agar, pork kidney/beef fat, chicken heart and intestine, clotted cattle blood, wheat, corn, fish, several fly media, and dog food (*2*, *4*, *5*, *13*; and R. A. Bedding, written communication). Almost any substance high in protein can be used for culturing *N. carpocapsae* as long as two criteria are met (*13*): first is the need for the continued association of the bacterium *Xenorhabdus nematophilus* (Poinar and Thomas) with the nematodes, and second is the necessity of keeping other micro-organisms out of the culture system. Dutky et al. (*3*) have shown that this nematode also requires an exogenous source of sterol for normal growth and reproduction.

One of the most economical media which satisfies the nematode's food requirements is dog food. House et al. (*5*) first reported the use of dog food for polyxenic (with several associated micro-organisms) mass production of *N. carpocapsae* (DD-136 strain), and various modifications of their technique are now in use. In this study, we describe another modification of the dog food method for the monoxenic (with one associated micro-organism) mass production of *N. carpocapsae* and methods of collection and storage of infective juveniles.

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[3]Italic numbers in parentheses refer to Literature Cited, p. 7.

MATERIALS AND METHODS

The All strain of *N. carpocapsae* was used in the mass production. The stock inoculum of *N. carpocapsae* and its associated bacterium were maintained on pork kidney/peptone agar medium in 16- by 150-mm culture tubes (*4*). This stock inoculum was initially obtained by surface sterilizing the infective juveniles using a modified technique developed by Lownsbery and Lownsbery (*11*) and Moody et al. (*12*). The apparatus for surface sterilization consisted of two separatory funnels (60 ml) and a vial (35 ml) as shown in figure 1. Several thousand infective juveniles were placed into a mixture of 0.013-percent Aretan (methoxyethyl mercury chloride) and 0.6-percent dihydrostreptomycin sulfate (U.S. Biochemical Corp., Cleveland, Ohio) in the upper separatory funnel. After 12 hr, the nematodes were delivered into the lower funnel for a sterile, distilled water rinse. After the nematodes settled, they were delivered into the vial containing sterile, distilled water. About 200 to 300 surface sterilized nematodes were placed onto the pork kidney/peptone agar medium. This method provided a monoxenic culture of nematodes for inoculation into the mass production scheme.

For mass production of the nematode, dog food chunks (Gravy Train, General Foods Corp., White Plains, N.Y.) were pulverized in a blender. One hundred grams of pulverized dog food were mixed with 500 ml of dissolved 1-percent agar. The dog food/agar medium was further blended for 1 min to obtain a uniform mixture and was autoclaved for 15 min at 123°C and 17 psi. After autoclaving, the dog food/agar medium was handled aseptically in the following procedure.

Upon cooling to about 60°C, 25-ml aliquots were poured into sterile plastic petri dishes and were inoculated with nematodes from the monoxenic stock culture by placing 6 ml of sterile distilled water into the culture tubes and pipetting about a 0.2-ml aliquot, containing 100 to 200 nematodes, onto the surface of the medium. Petri dishes were sealed with Parafilm (American Can Co., Greenwich, Conn.) and incubated at 25°C for 20 to 30 days before trapping. The Parafilm served to prevent rapid desiccation and fungal or mite contamination.

Trapping of the infective juveniles was accomplished by a modified method of Lindegren et al. (*10*). This method consisted of removing the cover of the petri dish and placing the petri dish bottom, containing the nematode-infested medium, into a larger plastic petri dish (150 by 25 mm). The larger petri dish contained 40 ml of 0.1-percent formalin into which the infective juveniles migrated over a bridge formed by sterilized filter papers (5.5 cm in diameter, Whatman No. 1) (figs. 2 and 3).

Infective juveniles were harvested from the nematode traps every other day for about 3 weeks by pouring the nematode suspension into a 400-ml beaker and allowing the nematodes to settle for 45 min. The supernatant was aspirated using a vacuum flask (fig. 4), and the nematodes were concentrated with a clinical centrifuge at 300 revolutions per minute for 1 min (fig. 5). The number of nematodes was calculated volumetrically, and the nematodes were transfered to plastic tissue culture flasks (150 cm^2) or petri dishes (150 by 15 mm) in 200 ml or 60 ml of 0.1-percent formalin, respectively. These were stored at 10°C at various densities from 30,000 to 170,000 nematodes per milliliter.

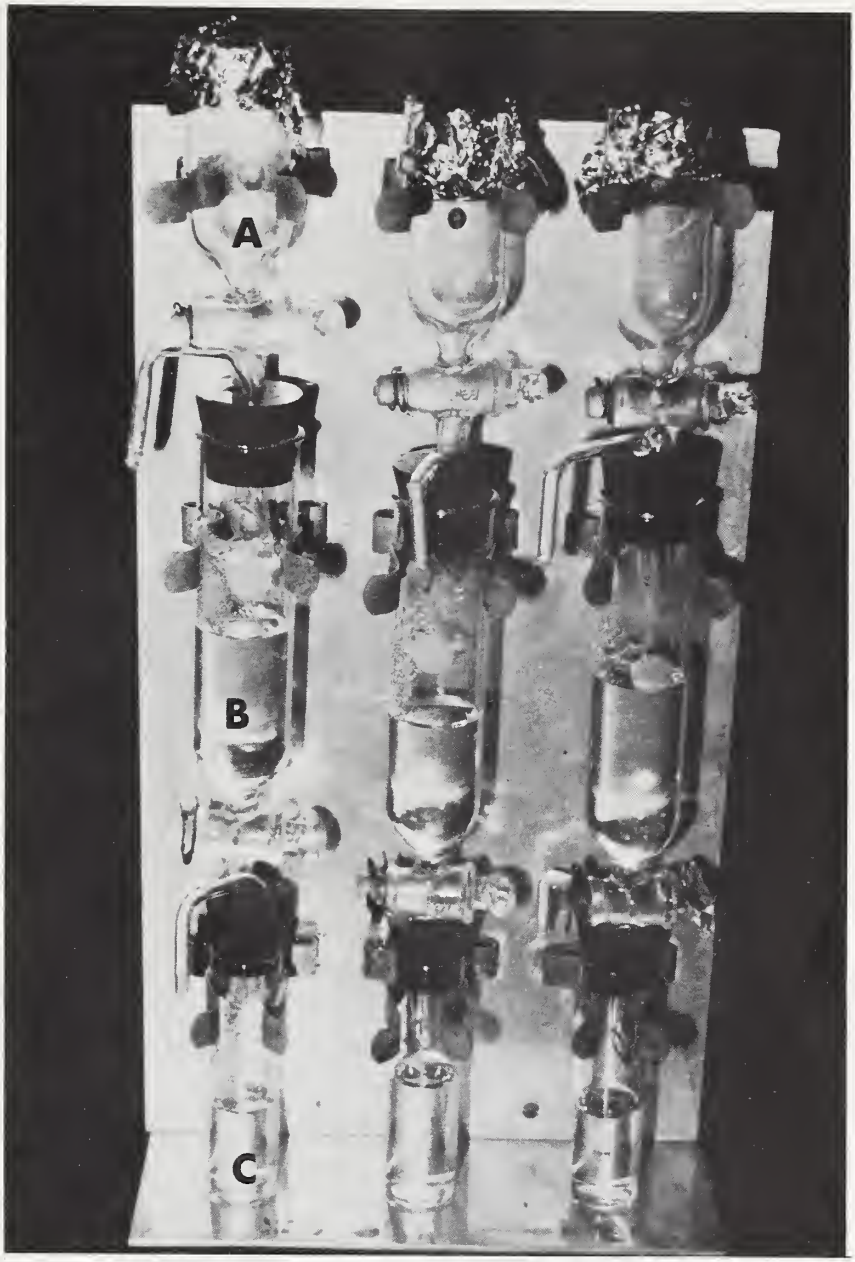

Figure 1.--Nematode sterilizing apparatus consists of a series of two separatory funnels (*A* and *B*) and a vial (*C*): *A*, Upper funnels contain 0.013-percent methoxyethyl mercury chloride and 0.6-percent dihydrostreptomycin sulfate; *B*, lower funnels are for sterile distilled water rinse; and *C*, nematodes are collected in vials containing sterile distilled water.

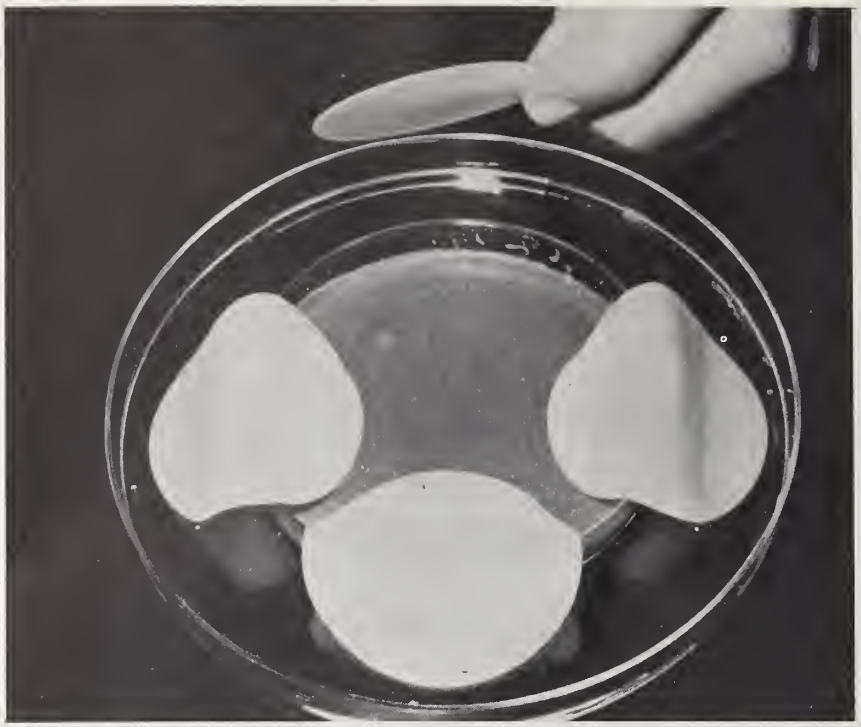

Figure 2.--Infective juveniles are trapped by placing the petri dish bottom, containing the nematodes, into a larger petri dish. The larger petri dish contains 40 ml of 0.1-percent formalin into which infective juveniles migrate over a bridge formed by filter papers.

Figure 3.--Mass trapping of nematodes, showing stacked petri dish traps.

Figure 4.--A vacuum flask aspirates supernatant from beaker containing settled nematodes.

Figure 5.--Clinical centrifuge used to concentrate nematodes.

After 30 and 180 days of storage, observations were made on nematode survival and infectivity. Survival was determined by response to mechanical stimulation with a probe. A nematode infectivity test was conducted on larvae of *Galleria mellonella* (L.), using a modification of Dutky's method (*4*, *8*).

RESULTS AND DISCUSSION

The mass production of *N. carpocapsae* produced 100,000 juveniles per gram of dog food/agar medium. Each petri dish averaged 400,000 juveniles per harvest and was harvested five to seven times over a 2- to 3-week period for a total average yield of 2 million juveniles per petri dish. The trapping method was effective in obtaining only the infective stage juveniles. Maximum yields were obtained during the first week of harvest. Over 125 million infective juveniles were collected weekly from 100 petri dishes by one technician working 20 hr per week. The technician's time included all aspects of the mass production.

Volumetric estimates of nematode numbers saved considerable time. One milliliter of concentrated infective juveniles contained about 1.8 million nematodes. Centrifugation in calibrated tubes did not reduce nematode survival or infectivity.

Initially, fungal and bacterial contamination of the nematode culture dish was a problem during the trapping phase of the production. This problem was resolved by allowing the nematodes to develop and reproduce for a minimum of 20 days on the dog food/agar medium. As the incubation period increased, the moisture content of the dog food/agar medium decreased, which favored the infective juveniles and also lessened fungal and bacterial contamination during the trapping phase.

The cost of mass production (labor, ingredients, and supplies) using the dog food/agar medium was about 28 cents per million nematodes. Labor accounted for 64 percent of the cost and the ingredients and supplies for 36 percent. By comparison, Poinar (*13*) reported that one technician at a commercial company (Nutrilite Products) could handle about 200 petri dishes of dog food medium a day and could collect 100 million nematodes each week at a cost of $1 per million. Bedding (*2*), using aspen wool coated with chicken heart homogenate in a monoxenic, three-dimensional culture system, produced 100 million nematodes per 2-L container. The total cost of rearing *N. carpocapsae* was about 2 cents per million nematodes, but Bedding encountered two major problems in this system. One was the highly variable yields of nematodes, and the other was a mixture of different nematode stages in the final suspension.

Subsequently, R. A. Bedding (written communication) modified the mass production system and used crumbled polyether polyurethane foam coated with pork kidney/beef fat homogenate in 500-ml Erlenmeyer flasks. He reported an average yield of 38 million infective juveniles per flask at a cost of less than 2 cents per million for *N. carpocapsae*.

Bedding's method is more conducive to commercial development than our mass production system; however, our system for *N. carpocapsae* is feasible for research laboratories because it uses a readily available medium (dog food) and requires no specialized equipment. A pressure cooker can replace the use of an autoclave. Our system has also been successfully used with *Neoaplectana glaseri* Steiner, and other strains of *N. carpocapsae*, including DD-136, Mexican, and Agriotos (*7*; Lindegren, unpublished data; and Kaya and Hara, unpublished data).

Recently, Akhurst (*1*) found that bacterial isolates of *Xenorhabdus nematophilus* from *N. carpocapsae* have two forms. The primary form is usually found in infective juveniles and is maintained or at least dominant for the first few days in the insect's hemocoel. Later, the secondary form may be produced within the host cadaver and is almost invariably present after a few days on artifical media. In the presence of the primary form, nematode reproduction is optimal but is greatly reduced when an abundance of the secondary form is present. R. A. Bedding (written communication) stressed that understanding the dimorphic character of the symbiotic bacteria is essential for successful mass culture of *Neaoplectana* spp.

The type of storage container or nematode density did not significantly affect nematode survival. After 180 days of storage, 95 to 100 percent of the nematodes survived, and these nematodes infected 100 percent of *Galleria mellonella* larvae. Lindegren et al. (*10*) found that infective juveniles stored in 150- by 15-mm plastic petri dishes at 6°C at about 160,000 individuals per milliliter had about 94-percent survival after 270 days of storage. For prolonged storage, Dutky et al. (*4*) stressed the importance of oxygenation and large surface area-to-volume

ratio for oxygen exchange. In our case, the plastic tissue culture flasks and petri dishes apparently provided the proper conditions for storage for the 180-day period. Dutky et al. (*4*) concluded that if the nematodes survived the first few weeks of storage, they would do so for a long time.

Another method that has been used for long term storage is nonliquid media. Howell (*6*) reported consistently higher survival when *N. carpocapsae* was stored at 3°C on formalin/water saturated filter paper for about one year. Prolonged storage (19 months), however, appeared to affect infectivity. Other methods of storage, such as lyophilization, are still in the experimental stage (*13*).

Our modification of the dog food/agar medium is an efficient system to mass produce pure suspensions of *N. carpocapsae* infective juveniles. This method has enabled extensive field testing of this nematode against the navel orangeworm, *Amyelois transitella* (Walker), the western spruce budworm, *Choristoneura occidentalis* (Freeman), and the elm leaf beetle, *Pyrrhalta luteola* (Muller) (*9*; and Kaya and Hara, unpublished data).

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